NEUROMUSCULAR TRANSMISSION AND CORRELATIVE MORPHOLOGY IN YOUNG AND OLD MICE

By BETTY Q. BANKER, S. S. KELLY* AND N. ROBBINS†

From the Departments of Anatomy and Pathology, Case Western Reserve School of Medicine, Cleveland, OH 44106, U.S.A.

SUMMARY

1. Age changes in spontaneous and evoked transmitter release, in receptor number and in ultrastructure at the neuromuscular junction were studied in the CBF-1 mouse strain, which stays physically active and relatively free of organ pathology into advanced age.

2. Spontaneous miniature end-plate potentials (m.e.p.p.s) were recorded in the following young (8–12 months) and old (29–33 months) mouse muscles: extensor digitorum longus (e.d.l.), soleus (sol.), gluteus maximus (g.m.), diaphragm (diaph.) and extensor digitorum communis (e.d.c.).

3. M.e.p.p. amplitudes were unchanged with age in four muscle groups despite increases in input resistance (in e.d.l., sol. and g.m.). M.e.p.p. amplitude in old diaph. increased 54 % with no change in input resistance. Bimodal distributions of m.e.p.p. amplitudes were observed in 6–23 % of muscle fibres but were not more prevalent in old mice. There was little or no change in resting membrane potential with age.

4. Numbers of junctional acetylcholine receptors (measured with ^{125}I-α-bungarotoxin) were the same in all young and old muscles except e.d.l., where a 30 % decrease was noted. Extrajunctional receptors and other indicators of denervation (decreases in resting potential, twitch tension or muscle fibre diameter) were absent or minimal.

5. M.e.p.p. frequency decreased in e.d.l., sol. and e.d.c. but not in g.m. or diaph. There was no correlated change in the cholinesterase-positive end-plate area.

6. It is concluded that m.e.p.p. amplitude is maintained in old muscles by a combination of compensatory changes. The decline in m.e.p.p. frequency varies between muscle groups and is independent of the length of the motoneurone axon or level of innervation.

7. Evoked end-plate potentials (e.p.p.s) were recorded in e.d.l., sol. and diaph. from young (11–13 months) and old (29–30 or 34–35 months) male CBF-1 mice in curarized preparations stimulated at 2 or 20 Hz. The amplitude of the initial e.p.p. of the trains was increased by 122 % in old e.d.l. and 93 % in old sol., and plateau e.p.p. amplitudes were also increased by about 100 % (e.d.l.) and 67 % (sol.). This, combined with the absence of change in m.e.p.p. amplitude with age, suggests that

* Present address: Department of Pharmacy, University of Aston, Birmingham B4 7ET.

† To whom reprint requests should be addressed.

the number of quanta released per nerve impulse was increased. In diaph. there was no change with age.

8. In all muscle groups, the threshold for initiation of the muscle action potential was unchanged with age. Thus, the relative safety factor of transmission was *increased* in curarized old e.d.l. and sol. (but not diaph.).

9. Depression of the indirect twitch in solutions with a decreased calcium: magnesium ratio was also used as a relative measure of synaptic efficacy. Old sol. and e.d.l. but not diaph. muscles showed less depression of indirect twitch amplitude than did young muscle under these conditions.

10. In cut-fibre preparations of sol. and diaph. stimulated at 20 Hz, there was no age-dependent difference in e.p.p. amplitude, in directly measured quantal content, or in curare sensitivity. In view of other results, these findings require careful interpretation.

11. Ultrastructural morphometry was carried out in e.d.l. The nerve terminals in old (30 and 34 months) e.d.l. muscles exhibited pronounced loss of synaptic vesicles. In 34-month animals, decreased nerve terminal area and post-synaptic folds devoid of nerve terminals were often observed. Since no evidence of denervation was found by physiological criteria, it is concluded that in 34-month mice, nerve terminals withdraw from some synaptic gutters but do not abandon any junction entirely. The large presynaptic ultrastructural changes contrast with the physiological data showing no deficit and even increases in transmitter release. Therefore, under these conditions, these profound structural changes are either not functionally significant or are well compensated.

INTRODUCTION

There is no comprehensive physiological information on ageing at any synapse despite hints in the literature that the aged synapse may reveal interesting adaptations. We have chosen the neuromuscular junction as a system in which to study synaptic ageing, especially in view of the growing body of knowledge of adaptational changes at the adult junction (Robbins, 1980; Grinnell & Herrera, 1981).

Information about neuromuscular transmission in aged animals is sparse. In aged rats, the safety factor, as indirectly indicated by curare sensitivity, was apparently reduced (Fischer & Birren, 1947; Smith, 1979) but no quantal analysis or characterization of end-plate potential amplitudes during tetanic trains has been published. Also, the correlation between natural pattern of activity and rate or character of physiological synaptic ageing remains to be explored. The most complete report to date (Smith, 1979) focussed on the rat diaphragm, which is a continuously active phasic muscle and which, in the rat, is less affected by ageing than limb muscles (Standaert & Booher, 1981). Therefore, we have examined neuromuscular transmission in three muscles of young and old mice: the extensor digitorum longus (a phasic muscle), the soleus (a tonic muscle), and the diaphragm. Miniature end-plate potentials (m.e.p.p.s) were also studied in two additional muscles. Particular muscle groups were selected to determine whether age changes were homogeneous in all junctions of a given muscle group, and whether the nature and severity of age changes were correlated with the rostrocaudal level of innervation, distal or proximal location in limb, or length of axon supplying the muscle. We also examined post-synaptic

parameters which are shown to affect the amplitude of the post-synaptic response. In particular, in view of conflicting or variable reported results in junctional acetylcholine receptors with age, (Pestronk, Drachman & Griffin, 1980; Courtney & Steinbach, 1981), the density of junctional and extrajunctional receptors were measured. A single fibre technique was used to obtain more accurate values, and five muscle types were examined to explore the generality of the findings.

To date, intracellular characterization of neuromuscular function with age has been confined to the rat, and there is reason to question the generality of the results obtained, either because of the previously unexamined organ pathology only now coming to light (e.g. Caccia, Harris & Johnson, 1979) or because of the extreme obesity and inactivity typical of the aging common laboratory rat. The profound reduction in m.e.p.p. frequency reported in such animals (Gutmann, Hanzlikova & Vyskocil, 1971) and the opposite finding in the special Fisher 344 strain (Smith, 1979) might reflect either of these abnormal conditions. We have found that the CBF-1 strain of mouse reaches an advanced age with infrequent pathological changes and with grossly well-maintained locomotor activity. Therefore, we chose these animals to characterize age changes in neuromuscular transmission. In addition, we have determined whether the ultrastructural changes associated with ageing at the neuro-muscular junction in the mouse (Fahim & Robbins, 1982) are accompanied by correspondingly altered synaptic transmission or signs of denervation, especially when electron microscopic analysis was carried out in the same batches of mice in which physiological experiments were performed.

Finally, the notion of partial denervation and reinnervation in aging synapses has been suggested frequently (Gutmann & Hanzlikova, 1965) without rigorous test. Therefore, a wide range of physiological and structural parameters known to change with denervation were examined.

METHODS

Male CBF-1 mice (CB6F, BALB/cNNia × c57BL/6NNia) were obtained from the Charles River Breeding Laboratory, and were either 8–12 months (young adult) or 29–33 months of age (old) except where otherwise stated. The mean body weights (37 g) of these two age groups were not significantly different. The mean life span (50 % survival point) in this strain is 29 months and 25 % survive to 34 months. Moribund mice or those with gross organ pathology were not used. The presence of distended seminal vesicles, commonly observed in old mice, was not taken as grounds for rejection.

Muscles were removed from animals under methoxyflurane (Pitman-Moore) anaesthesia, and were temporarily pinned onto Silgard 184 (Dow Corning) in 22–24 °C Krebs saline (pH 7·2) oxygenated with 95 % O_2–5 % CO_2. The composition of the saline was (mM): NaCl, 135; KCl, 5; $NaHCO_3$ 15; Ca gluconate, 2·5; $MgSO_4$, 1; Na_2HPO_4, 1; glucose, 11. A gas mixture of 95 % O_2 and 5 % CO_2 was bubbled through the saline for at least one hour before use.

Routine pathology

Samples of heart, lung, liver, kidney, adrenal, and spleen taken from all aged animals and from four young animals were fixed in formalin, embedded in paraffin and stained with haemotoxylin and eosin.

Electrophysiological recording

Muscles were placed in a Perspex chamber (volume *ca.* 10 ml.) through which oxygenated Krebs saline at 30·0 ± 0·5 °C flowed at a rate of 10–15 ml. per minute. Glass capillary micro-electrodes filled with 3 M-KCl were inserted into muscle fibres at the end-plate region in order to record m.e.p.p.s. Occasionally two electrodes were inserted into the same muscle fibre within 50 μm of each other, and

0.5×10^{-8} A or 1.25×10^{-8} A hyperpolarizing current was injected through one electrode while the voltage response was recorded from the other to determine muscle fibre input resistance. In similar experiments depolarizing current (5 msec duration) was increased while the voltage response and the resting membrane potential (r.m.p.) were monitored on a storage oscilloscope, and the voltage at which a regenerative action potential was initiated was taken as threshold for that muscle fibre. R.m.p.s were recorded on a Brush Mark 220 pen recorder and focal m.e.p.p.s (rise time < 1 msec) together with a 1 mV calibration pulse were recorded on a Hewlett–Packard 3964A instrumentation recorder. Usually, 100–300 m.e.p.p.s were recorded from each muscle fibre and for each muscle, twenty-eight to forty fibres were sampled from four or five mice in each group.

Recorded m.e.p.p.s were played back via an analogue to digital converter into a PDP 11/23 minicomputer which was used to correct amplitudes to a standard r.m.p. of -80 mV (Kelly, 1978), to calculate the mean amplitude and frequency of m.e.p.p.s at each end-plate, and to plot amplitude and frequency distribution histograms via a Hewlett–Packard 7221A plotter. In any muscle fibre, 'giant' m.e.p.p.s were defined as those of amplitude greater than twice the mean or of duration greater than 10 msec. Such 'giants' were not used in the calculations of mean amplitude and frequency. The over-all mean amplitude and frequency in any one muscle group was calculated from the individual means of the fibres in that group and differences between means were considered to be significant if the Mann–Whitney non-parametric test gave P values less than 0.05. Student's t test (two-tailed) was used only for bungarotoxin binding results in which the number of observations was less than ten.

In most experiments (as indicated), end-plate potentials (e.p.p.s) were recorded in the standard saline containing 2.6 μM (+)-tubocurarine chloride (curare). Extensor digitorum longus (e.d.l.), soleus (sol.), and diaphragm (diaph.) muscles were used. The nerves supplying the muscles were stimulated via a suction electrode using a WPI type 302-T anapulse stimulator and stimulus isolation unit. Stimuli were of 50 μsec duration and 3–5 times threshold intensity.

R.m.p.s. and trains of e.p.p.s, elicited by stimulating the nerve at either 2 Hz or 20 Hz, were recorded intracellularly from end-plate regions of muscle fibres. An end-plate region was taken to be a site at which the rate of rise of the e.p.p.s was less than 1 msec. Three trains of fifty e.p.p.s were usually recorded at each frequency from each muscle fibre. The e.p.p. amplitudes were corrected for non-linear summation (Martin, 1955) and to a standard r.m.p. of -80 mV (cf. Kelly, 1978) and the mean and variance of the last forty e.p.p.s were calculated. Any train in which there was a drift in mean amplitude between e.p.p.s 11 and 50 was discarded. The ratio of each of the first six e.p.p.s to the first e.p.p. in each train was calculated. The mean value of each parameter was determined for each muscle fibre and the resulting fibre mean was used to calculate the over-all mean for each muscle group. Occasionally, the refractory period of the nerve fibres was determined by slowly decreasing the interval between pairs of supramaximal stimuli until no e.p.p. could be elicited by the second stimulus.

Cut-fibre preparations were as described by Hubbard & Wilson (1973) except that the muscle was cut in normal Krebs solution. The m.e.p.p. and e.p.p. amplitudes were analysed as in the curare experiments, and the mean quantum content determined directly by dividing mean e.p.p. amplitude by mean m.e.p.p. amplitude. Given that the reversal potential in cut-fibre preparations was approximately -4 mV, Martin's correction for non-linear summation was empirically trimmed by a factor of 0.7 (see Discussion, McLachlan & Martin, 1981) so that corrected e.p.p. amplitudes would not exceed the reversal potential. E.p.p.s in both young and old muscles were corrected to a standardized resting potential for comparison.

The indirect twitch was also monitored as an index of synaptic depression. Nerve–muscle preparations were placed in a small dish with oxygenated Krebs solution at room temperature (22–24 °C), and either the proximal tendon (sol., e.d.l.) or the costal margin (diaph.) was firmly pinned to a silastic mount. The tendinous insertion was attached to a Statham UC-3 tension transducer, and the output was displayed on and measured from a storage oscilloscope. The nerve was stimulated via a suction electrode at twice maximal stimulus strength and initial tension was adjusted for maximum indirect twitch output. The Krebs solution was then replaced with a Krebs solution of reduced calcium:magnesium ratio (details given later), exchanged once again at 5 min, and the twitch tension tested after a total of 25 min in the altered solution. Lastly, the muscle was allowed to recover in normal Krebs solution. The results were accepted if the twitch tension returned to at least 85 % of the starting value. In computing percentage depression in the altered Krebs solution, the average of starting and finishing twitch tensions were used for normalization.

Electron microscopy

E.d.l. muscles from two 30 month and two 34 month old male CBF-1 mice were compared with the same muscle in two 4 and 5 month old mice. The animals were lightly anaesthetized by intraperitoneal injections of sodium pentobarbitone and the exposed muscle was bathed *in situ* for 5 min with 3·5 % glutaraldehyde in 0·1 M-cacodylate (pH 7·4). After 2 hr of fixation and buffer wash, the tissue was post-fixed in 2 % osmium tetroxide for 2 hr, dehydrated and embedded in epon. For morphometric study, the techniques of Engel, Tsujihata & Jerusalem (1975) were employed. All end-plate regions observed by electron microscopy were photographed and all photographs were analysed.

Single muscle fibre analyses

Methods of muscle dissociation, cholinesterase staining, bungarotoxin binding and quantitation are described in detail and evaluated elsewhere (Robbins, Olek, Kelly, Takach & Christopher, 1980). Since perijunctional counts were negligible (see Results), the end-plate counts gave directly the number of sites per end-plate. Because of the short lengths of fibre involved, the non-specific binding was also negligible compared to that of the end-plate.

<div align="center">RESULTS</div>

Pathology

In both young and old CBF-1 mice, routine histological examination of kidney, lungs, liver, heart, spleen and adrenal revealed only changes generally characteristic of ageing (Kohn, 1978). Occasional mice with gross pathology (liver or lung tumours, skin abcesses) were not used.

Physiology

M.e.p.p. amplitude. At most end-plates of young and old animals, the distribution of m.e.p.p. amplitudes was unimodal and the coefficient of variation was in the same range as that found in other studies of m.e.p.p. amplitudes (e.g. Harris & Ribchester, 1979; Vyskocil & Gutmann, 1972). The proportion of end-plates showing bimodal distribution in any one muscle group varied from 6 to 23 % and did not appear to change consistently with age. A more detailed study of these bimodal m.e.p.p.s will be reported elsewhere. In populations of fibres within any one muscle group, the distribution of mean m.e.p.p. amplitudes was always unimodal although often positively skewed (Fig. 1).

The mean m.e.p.p. amplitude changed with age only in the diaph. in which there was an increase (Table 1). There was no indication that the increased amplitude in the diaph. was due to a decrease in cholinesterase activity, since m.e.p.p. rise times and half decay times were the same in both young and old muscles. The increase in mean m.e.p.p. amplitude in old diaphragms resulted from a shift in the entire population of recorded junctions.

In a separate group of experiments, m.e.p.p.s were recorded in 34 month mice. In all three muscles – sol. (two mice, fifteen fibres), e.d.l. (two mice, seventeen fibres), and diaph. (four mice, thirty-six fibres) – m.e.p.p. amplitudes were not significantly different from those in 28–33 month animals.

The number of 'giants' per 100 m.e.p.p.s was unchanged with age except in diaph. where it was decreased. However, the frequency of 'giants' in *old* diaph. was similar to that found in all other young and old muscle groups (Table 1) and the large 'giants' in young rat diaph. are mostly eliminated by addition of tetrodotoxin (N. Robbins,

unpublished), so that spontaneous action potential in small nerve branches at the end-plate may account for the high frequency of 'giants' in young mouse diaph.

Several parameters known to affect m.e.p.p. amplitude were also examined.

Resting membrane potential. R.m.p. changed by 8 % or less with age (Table 1) and therefore would not affect m.e.p.p. amplitude significantly.
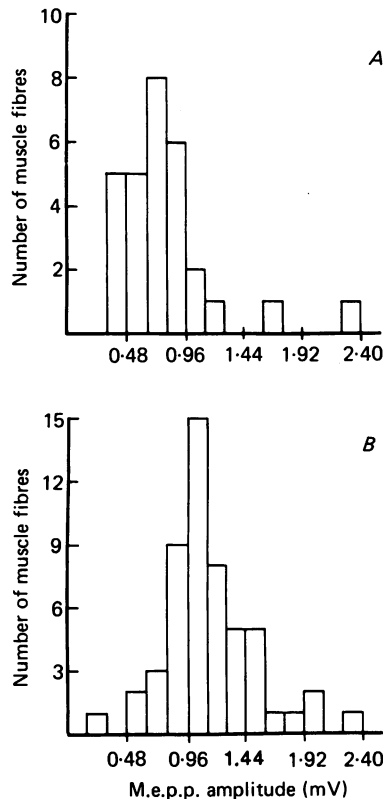

Fig. 1. Typical histograms of the distribution of mean m.e.p.p. amplitudes from muscle fibres within the same muscle group in diaph. from young (*A*) and old (*B*) mice.

Input resistance. With the exception of the diaph., there was a significant increase in input resistance between young and old animals, the largest increase being in the gluteus maximus (g.m.) (Table 1). M.e.p.p. amplitude has been found to be directly proportional to input resistance (Katz & Thesleff, 1957; Harris & Ribchester, 1979). However, these age-related changes in input resistance were not accompanied by changes in m.e.p.p. amplitude. Gage & McBurney (1973) have emphasized the importance of membrane capacitance in determining m.e.p.p. amplitude, but this was unchanged in old animals (Table 1).

Fibre diameter. Except for a small difference in the CBF-1 soleus muscle, fibre diameters did not change with age (Table 1). Histograms of fibre diameter distribution showed no shift within any particular muscle between young and old mice (e.g. Fig. 2). Diameters measured from unselected cross-sections of fresh-frozen muscle also

TABLE 1. Pre- and post-synaptic parameters in young and old muscles†

Muscle	Age (months)	R.m.p. (mV)	M.e.p.p. amplitude (mV)	M.e.p.p. frequency (Hz)	Input resistance (kΩ)	Membrane capacitance (nF)	Fibre diameter (μm)	BTX binding (×10⁷ sites/ end-plate)	End-plate area (μm²)
E.d.l.	8–12 Y	81·9±0·8 (37)	0·43±0·03 (37)	5·91±0·71 (37)	282±24 (22)	2·39±0·15 (22)	31·7±1·3 (34)	3·58±0·31 (5)	689±42 (37)
	28–33 O	81·3±1·3 (35)	0·47±0·03 (35)	3·99±0·71 (35)*	343±28 (15)*	2·74±0·41 (15)	32·5±1·8 (30)	2·37±0·49 (4)*	656±45 (26)
Sol.	8–12 Y	76·4±1·3 (33)	0·58±0·02 (33)	3·21±0·55 (33)	428±31 (20)	2·24±0·16 (19)	33·6±1·3 (41)	2·98±0·86 (4)	579±53 (17)
	28–33 O	78·3±1·2 (35)	0·53±0·03 (35)	1·81±0·25 (35)*	523±46 (18)*	2·00±0·14 (18)	28·5±1·3 (30)*	2·50±0·64 (4)	613±52 (15)
G.m.	8–12 Y	78·3±0·9 (33)	0·75±0·08 (33)	4·65±0·63 (33)	286±21 (35)	1·93±0·11 (18)	32·6±1·8 (35)	2·59±0·65 (3)	786±48 (24)
	28–33 O	81·4±0·9 (34)*	0·61±0·05 (34)	4·89±0·66 (34)	403±29 (18)*	1·60±0·16 (18)	35·6±2·2 (36)	2·91±0·59 (4)	672±38 (36)
E.d.c.	8–12 Y	81·8±0·6 (37)	0·60±0·04 (37)	4·07±0·52 (37)	—	—	25·4±1·4 (37)	2·00±0·43 (3)	499±42 (15)
	28–33 O	78·9±0·9 (37)*	0·66±0·05 (37)	2·93±0·27 (37)*	—	—	27·5±1·4 (34)	1·96±0·51 (3)	537±41 (18)
Diaph.	8–12 Y	70·0±1·5 (28)	0·74±0·05 (28)	1·37±0·20 (28)	696±51 (21)	1·10±0·15 (21)	—	1·04±0·18 (3)	—
	31–35 O	75·6±0·9 (53)*	1·16±0·05 (53)*	1·58±0·13 (53)	774±59 (20)	1·39±0·12 (20)	—	0·97±0·14 (3)	—

† All values are means ±s.e. derived from muscle fibres (numbers in parentheses) studied in three to six male CBF-1 mice. Asterisk indicates significant difference between young and old. For diaph. two age groups of old animals were used, 31 months (three animals, seventeen fibres) and 34–35 months old (four animals, thirty-six fibres) but because there was no significant difference between results from the two groups, the results have been combined.

showed no changes with age. These results are consistent with the finding that whole muscle weights of e.d.l. or sol. were not significantly different between young adult and old animals.

α-Bungarotoxin binding. ^{125}I-α-bungarotoxin binding in the end-plate region of innervated muscles provides a measure of the number of junctional acetylcholine receptors (Fambrough, 1979) provided there is no substantial perijunctional binding. In all young and old muscles, toxin-binding in perijunctional muscle segments (extending from about 70–210 μm from the end-plate) was no greater than background
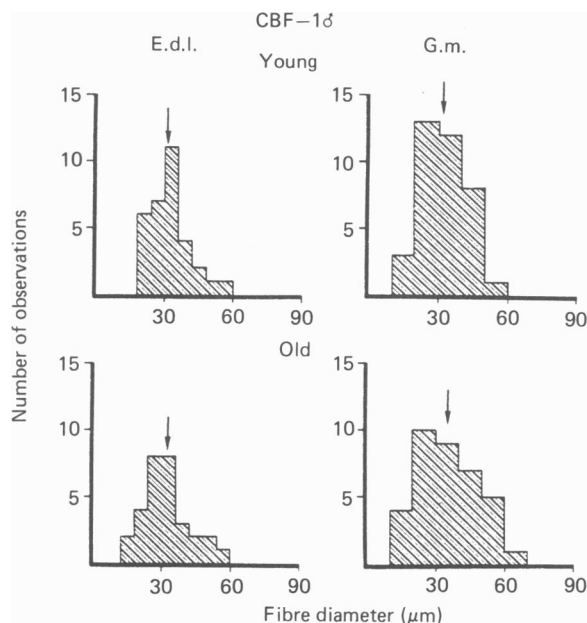

Fig. 2. Histograms of muscle fibre diameter distribution in e.d.l. and g.m. muscles of young and old CBF-1 mice. Arrow indicates mean diameter. See Table 2 for corresponding quantitative data.

(50–100 counts/min). End-plate binding was generally similar in young and old mice (Table 1). The exception was the e.d.l. of old CBF-1 mice, in which the number of binding sites was significantly reduced with age, whether expressed as absolute number of sites per end-plate or as average density (number of sites per end-plate divided by cholinesterase-positive end-plate area).

M.e.p.p. frequency. The distribution of m.e.p.p. frequencies between muscle fibres within the same muscle group was unimodal but often positively skewed. In e.d.l., sol., and extensor digitorum communis (e.d.c.) but not in gluteus maximus (g.m.) and diaph., m.e.p.p. frequency decreased with age (Table 1). Histograms of m.e.p.p. frequencies in any distal muscle group showed a downward shift with age in the whole population. Since only the distal muscles (e.d.l., sol., and e.d.c.) showed major age changes in m.e.p.p. frequency, a still more distal muscle, the flexor digitorum brevis, was examined, but no age-related change was found (1·72±0·16 Hz in young (twenty-six fibres in two muscles) *vs.* 1·68±0·18 Hz in old, forty fibres in three

muscles). Therefore, factors other than distal or proximal location are involved in alterations of m.e.p.p. frequency with age.

One possible explanation for reduced m.e.p.p. frequency would be a smaller end-plate area, since this has been correlated with reduced m.e.p.p. frequency (Harris & Ribchester, 1979). However, cholinesterase-positive end-plate areas were also unchanged with age (Table 1). Since only fibres in which the entire end-plate outline was visible were selected for analysis, the mean diameters of these selected fibres were greater than those of the randomly selected population (column 8, Table 1) in four out of eight muscle groups. This bias could not have affected the over-all result, because fibre diameter distribution (cf. Fig. 2) was unchanged with age and because in this limited range of diameters, there was no significant correlation between fibre diameter and end-plate area.

End-plates of young or old muscles having the same area might differ in the degree of complexity, another finding associated with altered m.e.p.p. frequency (Harris & Ribchester, 1979). However, inspection of experimental material showed no large difference in this respect. Furthermore, since end-plate area was unchanged with age (Table 1), a decrease in tortuosity or complexity would be manifest as a decrease in end-plate perimeter, but no change was found.

M.e.p.p. frequencies were also examined in a small number of 34 month mice (cf. m.e.p.p. amplitude results). In diaph. and in most sol. fibres, there was no difference from data presented above in 31 month mice but in a few old sol. fibres, values ranged from 4·8 Hz to 6·5 Hz. In e.d.l., m.e.p.p. frequency ($6·4 \pm 1·1$ Hz; $n = 17$) was higher than that in 28–33 month animals, and not significantly different from that in young mice.

E.p.p. amplitude

In e.d.l. and sol. muscles from old (34–35 month) mice, there was a pronounced increase in the amplitudes of the first e.p.p. of a train (Fig. 3) as well as the mean amplitude of plateau e.p.p.s (e.p.p.s 11–50) at both 2 Hz and 20 Hz (Table 2). This increase in e.p.p. amplitude with age was caused by a shift in the whole population (e.g. Fig. 4). The diaph. showed no change with age in either first or plateau e.p.p. amplitude.

In the e.d.l., plateau e.p.p. amplitudes at both 2 Hz and 20 Hz were increased in the same proportion as was the first e.p.p. In the old sol., synaptic depression at the beginning of the train was greater. At 2 Hz, but not at 20 Hz, this greater depression began as early as the second e.p.p. which, when expressed as a fraction of the first e.p.p., was significantly smaller (*ca.* 10 %) in the older animals (Fig. 5).

The rundown of e.p.p. amplitudes at the beginning of short trains at 2 Hz was modelled as an exponential decline using regression analysis. A good fit to an exponential decay was obtained by subtracting the mean amplitude of plateau e.p.p.s from the amplitude of each of the first six e.p.p.s. Such an analysis gave similar mean ±S.E. values of $-0·99 \pm 0·10$ ($n = 17$) and $-1·05 \pm 0·11$ ($n = 14$) for the exponential decay constants in young and old sol., respectively. In old sol., e.p.p. amplitudes ran down proportionately further than in young sol. but did not run down more steeply. There was no age difference in the mean exponential decay constants in e.d.l. or diaph.
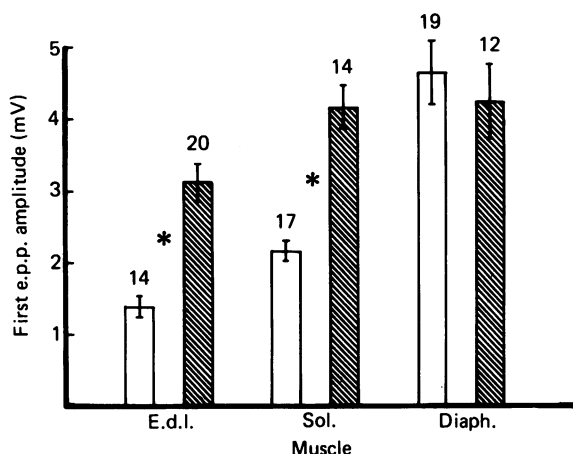
Fig. 3. Mean amplitudes of the first e.p.p. of trains in e.d.l., sol., and diaph. from young (Y) and old (O) male CBF-1 mice. Open and hatched bars are values in young and old animals, respectively. The number above each bar is the number of muscle fibres sampled, and the horizontal bars show ± 1 s.e. of mean. Asterisks indicate significant differences between young and old ($P < 0.05$).
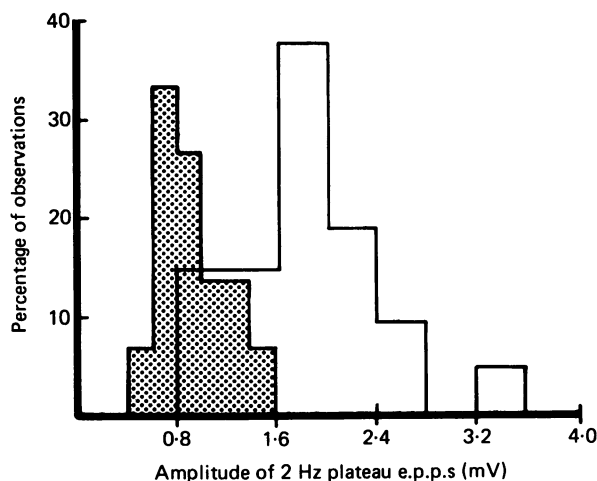

Fig. 4. Histograms showing the distribution of mean amplitudes of 2 Hz plateau e.p.p.s between e.d.l. muscle fibres in young (shaded histogram) and old (open histogram) male CBF-1 mice. The total number of fibres sampled was fifteen from young e.d.l. and twenty-one from old e.d.l.

Plateau e.p.p. quantum content

The reciprocal of the square of the coefficient of variation (i.e. $1/(CV)^2$ was calculated in curarized preparations to illustrate how the variance changes with the mean, because this should be proportional to quantum content both for Poisson and binomial statistics. There was general agreement between $1/(CV)^2$ and e.p.p. amplitude (Table 2), particularly in the comparison between young and old muscles (34–35 month) at a given frequency or between different frequencies in the same

TABLE 2. Muscle fibre threshold potential and e.p.p. parameter in e.d.l., sol., and diaph. from young (Y) and old (O) male CBF-1 mice (three to six mice in each group)

Parameter	Muscle					
	E.d.l.		Sol.		Diaph.	
	Y	O	Y	O	Y	O
Threshold (mV)	52·5±0·8 (22)	54·5±0·7 (21)	54·1±1·3 (16)	57·3±1·3 (18)	48·9±3·0 (8)	53·8±1·0 (9)
2 Hz plateau e.p.p. (mV)	0·92±0·08 (15)*	1·88±0·13 (21)	1·23±0·09 (17)*	2·04±0·13 (15)	2·37±0·21 (19)	2·25±0·27 (12)
$1/(CV)^2$ (2 Hz)	216±17 (15)*	328±38 (21)	176±17 (17)	230±23 (15)	113±12 (19)	105±16 (12)
Plateau e.p.p./first e.p.p. (2 Hz)	0·65±0·01 (14)	0·63±0·02 (20)	0·57±0·01 (17)*	0·50±0·02 (14)	0·52±0·02 (19)	0·54±0·01 (12)
20 Hz plateau e.p.p. (mV)	0·65±0·08 (12)*	1·28±0·09 (20)	0·75±0·04 (17)*	1·27±0·09 (12)	1·61±0·12 (17)	1·22±0·15 (8)
$1/(CV)^2$ (20 Hz)	160±19 (12)*	245±21 (20)	109±10 (17)*	193±45 (11)	97±15 (16)	85±20 (8)
Plateau e.p.p./first e.p.p. (20 Hz)	0·46±0·02 (12)	0·43±0·01 (20)	0·36±0·01 (17)*	0·31±0·01 (11)	0·36±0·02 (16)	0·37±0·03 (7)
Plateau (20 Hz)/plateau (2 Hz)	0·66±0·03 (10)	0·69±0·02 (17)	0·64±0·02 (10)	0·61±0·02 (10)	0·72±0·02 (12)	0·69±0·05 (6)

Values are mean ±1 s.e. of mean, with the number of muscle fibres in parentheses. Asterisks indicate significant difference between young and old ($P < 0.05$).

muscle. In the old sol. the large variability of the calculated $1/(CV)^2$ may account for the fact that the difference between mean values of the two age groups was not significant at 2 Hz, and was so at 20 Hz. The diaph., which showed no change in e.p.p. amplitude, also showed no change in $1/(CV)^2$. In another series of young (10 month) and old (30 month) animals, the same proportionate results were obtained (fibres from four muscles per group were analysed).
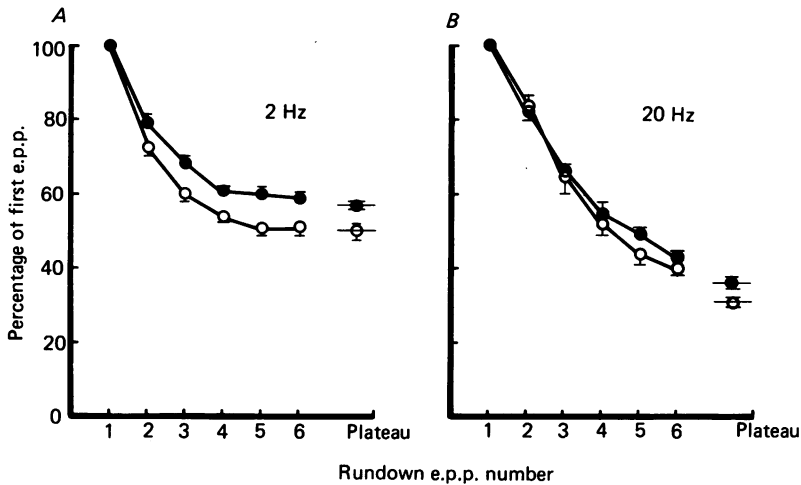

Fig. 5. The first six and plateau e.p.p.s of trains at A, 2 Hz and B, 20 Hz in sol. from young (●) and old (○) male CBF-1 mice. Values are expressed as ratio to the first e.p.p. and horizontal bars show ±s.e. of mean.

Safety factor

Assuming that curare reduces quantum size by the same proportion in young and old muscles, and that its presynaptic effect (if any) is the same in both age groups, it is possible to calculate the *relative* change in safety factor with age (cf. Kelly, 1976, 1978). However, these values give no indication of the absolute safety factor (s.f.). If the safety factor is defined as the amount of depolarization produced by an e.p.p. divided by that required to reach threshold, then the s.f. is given by:

$$\text{s.f.} = \frac{\text{m.e.p.p. amplitude} \times \text{quantum content}}{\text{corr (r.m.p.} - \text{threshold potential)}}$$

where corr (r.m.p. − threshold) is the amount of depolarization required to reach threshold, after correction for non-linear summation (Martin, 1955). If curare reduces the m.e.p.p. amplitude to a fraction, F, then:

$$\text{e.p.p.} = \text{F(m.e.p.p. amplitude} \times \text{quantum content)},$$

where e.p.p. is the corrected e.p.p. amplitude in curare

$$\text{F} \times \text{s.f.} = \frac{\text{e.p.p.}}{\text{corr(r.m.p.} - \text{threshold)}}$$

and

$$\frac{\text{s.f.}_2}{\text{s.f.}_1} = \frac{\text{e.p.p.}_2 \times \text{corr(r.m.p.}_1 - \text{threshold}_1)}{\text{e.p.p.}_1 \times \text{corr(r.m.p.}_2 - \text{threshold}_2)}$$

where s.f.$_1$ and s.f.$_2$ are the safety factors of muscle group 1 and 2, respectively. All the terms on the right hand side of the equation are measurable. The safety factor increased with age in e.d.l. and sol. whereas the diaph. showed no change (Fig. 6). The changes in e.d.l. and sol. were due mainly to altered e.p.p. amplitude (see Tables 1 and 2 for r.m.p. and threshold data, respectively).
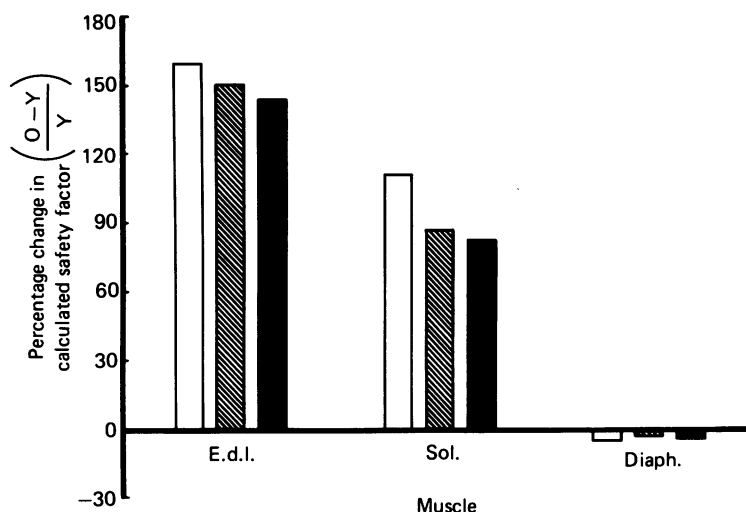

Fig. 6. Bar chart illustrating the percentage change $(O - Y)/(Y)$ in calculated safety factor with age in e.d.l., sol. and diaph. from male CBF-1 mice. Open bars, hatched bars and filled bars represent safety factors of first e.p.p., 2 Hz plateau e.p.p. and 20 Hz plateau. e.p.p., respectively.

Indirect twitch in solutions with altered calcium or magnesium

To estimate synaptic transmitter output averaged over the entire population of junctions in young and old muscles, the indirect twitch was measured in sol., e.d.l., and diaph. under conditions of reduced calcium: magnesium ratio. Under these conditions, transmitter release is reduced (e.g. Del Castillo & Katz, 1954) whereas the direct twitch is unaffected (e.g. Grinnell & Herrera, 1980); this was confirmed in two to four young and old muscles of each type. Therefore, reduction in amplitude of the indirect twitch gives a relative measure of synaptic efficacy. Young e.d.l. and sol. muscles showed significantly greater depression of twitch amplitude than did old muscles from 28–31 month old mice (Fig. 7). In contrast, young and old diaph. showed no difference in twitch reduction. These results are similar to those obtained in curarized muscle.

Transmitter release in cut-fibre preparations

In order to estimate transmitter release in normal external medium and in the absence of a drug such as curare, cut-fibre preparations were studied in young and old (28–30 months) sol. and diaph. muscles. When muscles were cut sufficiently to prevent twitching, resting membrane potentials and e.p.p. reversal potentials were similar in young and old sol. and diaph. muscles (Table 3). Amplitudes at the

beginning (e.p.p.$_1$) and during the plateau of a 20 Hz train were also identical in young and old muscles (Table 3). The direct quantal content of each junction, measured by dividing mean plateau e.p.p. amplitude by mean m.e.p.p. amplitude, was apparently the same in young and old junctions (Table 3). The apparent discrepancy between these and the preceeding data will be discussed. To test for differential curare
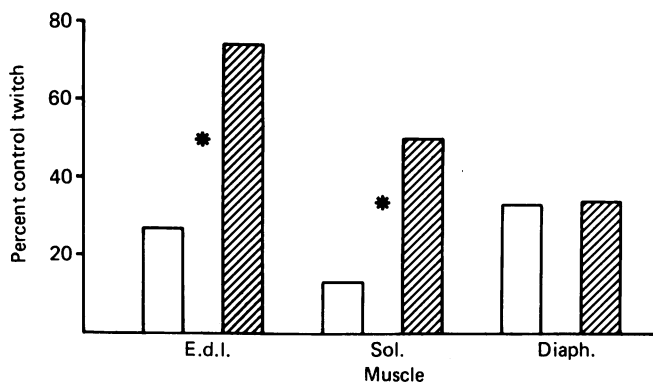

Fig. 7. Depression of indirect twitch amplitudes of young and old muscles in solutions with altered Ca and Mg. Results expressed as percent of twitch amplitude in normal Krebs solution. Ca/Mg concentrations (mM) were: 0·75/1·5 e.d.l., 0·5/1·75 sol., and 0·5/2·0 diaph. Data from five to seven muscles for each bar. * Ratios of young (□) and old (■) significantly different ($P < 0·05$). No s.D. bars are given because of use of log transformation in computing mean (Snedecor & Cochran, 1980). Muscles from a few old animals with small non-malignant lung adenomas were included in these data.

TABLE 3. Synaptic transmission in cut-fibre preparations of young (Y) and old (O) sol. and diaph.†

	Sol.		Diaph.	
	Y	O	Y	O
No. fibres (no. mice)	20 (3)	22 (4)	17 (3)	15 (3)
Resting membrane potential (mV)	−50·0±1·7	−49·4±1·5	−44·0±2·0	−42·7±1·3
M.e.p.p. amplitude (mV)	0·44±0·03	0·42±0·03	0·62±0·06	0·61±0·04
E.p.p. reversal potential (mV)‡	−4·1±1·1	−3·5±1·1	−3·6±1·4	−5·1±0·6
E.p.p.$_1$ amplitude	36·3±1·9	37·2±2·7	27·4±2·2	24·8±2·7
Apparent quantal content (m) (E.p.p. plat./m.e.p.p.)	55·9±4·3	58·7±3·4	32·6±3·1	34·5±2·9
(E.p.p. coefficient variation)$^{-2}$	371±48	303±38	259±41	333±53

  † Data are means ±s.E. of mean. All m.e.p.p. and e.p.p. data were corrected both for non-linear summation and to a resting potential of −50 mV (sol.) or −44 mV (Diaph.): see Methods.
  ‡ Data from eight to ten fibres per muscle type.

sensitivity in young and old junctions, a series of e.p.p. amplitudes were obtained in different muscle fibres before and 25–50 min after exposure to 2 μM + -tubocurarine. The ratio of e.p.p. amplitudes, ((before curare)/(after curare)), varied considerably between animals of the same age, but the range was similar in young and old muscles (ratios: 7·4–12·0 young sol.; 7·5–8·0 old sol.; 7·4–8·4 young diaph.; 7·8 one old diaph.). Therefore, curare sensitivity appeared unchanged with age.

Ultrastructural characterization

Quantitative analysis of conformational changes at motor end-plate regions of old e.d.l. muscles showed a number of alterations common to both 30 and 34 month old animals (Table 4). These included focal atrophy of post-synaptic folds and nerve terminal retraction from the synaptic folds. Very often, Schwann cytoplasm extended into the primary cleft in these zones and resulted in wide separation of the nerve terminal from the synaptic fold (compare Pl. 1 *A* and 1 *B*). Post-synaptic deposition of lipofuchsin was conspicuous in almost half of the fibres examined.

TABLE 4. Conformational ultrastructural changes at the motor end-plate as a function of age

| | E.d.l. muscle | E.d.l. muscle | |
Age (months)...	7–11	30	34
Number of motor end-plates studied	24	15	20
Number of motor end-plate regions studied*	60	56	38
Alterations at motor end-plate regions	No. of regions		
Focal atrophy of post-synaptic folds	0	14	15
Retraction of nerve terminal	0	0	2
Retraction of nerve terminal with interposition of basement membrane	0	0	1
Retraction of nerve terminal with interposition of Schwann cytoplasm	0	8	12
Terminal axon atrophy	0	2	4
Nerve terminal absence	0	2	15
Engorgement of nerve terminal with neurofilaments and neurotubules	0	34	0
Autophagic vacuoles within nerve terminal	0	0	9
Confluence of axoplasm within nerve terminal	0	0	11
Alterations at entire motor end-plate	No. of end-plates		
Abnormal pre-terminal nerve	0	1	7
Normal pre-terminal nerve	10	1	2
Lipofuchsin deposition within Schwann cytoplasm	0	0	1
Post-synaptic deposition of lipofuchsin	0	6	9

* Each motor end-plate contains multiple regions. Each region consists of a nerve terminal, a primary synaptic cleft, secondary synaptic clefts, junctional folds and a cover of Schwann cytoplasm. All regions of a single motor end-plate are in close association, but number of regions and the distances between them are variable.

Other conformational changes appeared to be progressive with age from 30 to 34 months (Table 4), e.g. absence of nerve terminals from individual regions increasing from 4 to 39 %. In five blocks where more than two regions were examined at a given motor end-plate, only one showed complete absence of nerve terminals at all regions. Thus, there appears to be piecemeal withdrawal of individual nerve terminals rather than complete denervation of an entire junction (see below). In the one instance in which all five regions demonstrated absent nerve terminals, the preterminal axon had degenerated. In general, abnormal preterminal nerves were more prevalent in the 34 month than in the 30 month age group. The converse was true with respect to engorgement of nerve terminals with neurofilaments (Table 4).

Morphometric analysis revealed a 69 % decline in the number of-synaptic vesicles per unit nerve terminal area in 30 month animals, although nerve terminal areas had not changed (Table 5; examples in Pls. 1 and 2). By 34 months, nerve terminal atrophy was reflected in a decreased terminal area, and possibly as a result, mean vesicle and mitochondrial density were not significantly different from that of young mice (Table 4).

TABLE 5. Electron microscopic morphometric data: motor end-plates in extensor digitorum longus in young and old mice

	Young	Old CBF-1	
Age (months)	7 – 11	30	34
Number of end-plates studied	24	15	20
Number of end-plate regions	60	54	38
Pre-synaptic region of motor end-plate			
Nerve terminal length (μm²)	$6\cdot05\pm0\cdot48$	$4\cdot89\pm0\cdot45$	$4\cdot27\pm0\cdot52$
Nerve terminal area (μm²)	$5\cdot09\pm0\cdot58$	$4\cdot64\pm0\cdot65$	$1\cdot93\pm0\cdot29*$
Vesicles per nerve terminal area (number/μm²)	$75\cdot00\pm4\cdot34$	$23\cdot65\pm1\cdot82*$	$67\cdot65\pm7\cdot03$
Mitochondrial area (%)	$12\cdot19\pm1\cdot16$	$10\cdot02\pm1\cdot14$	$13\cdot20\pm1\cdot79$
Absolute area of mitochondria (μm²)	$0\cdot66\pm0\cdot08$	$0\cdot50\pm0\cdot08$	$0\cdot33\pm0\cdot07$
Post-synaptic region of motor end-plate			
Post-synaptic area per nerve terminal (μm²)	$5\cdot21\pm0\cdot37$	$4\cdot01\pm0\cdot45$	$4\cdot87\pm0\cdot42$
Membrane profile concentration (μm/μm²)	$5\cdot89\pm0\cdot16$	$6\cdot87\pm0\cdot84$	$5\cdot95\pm0\cdot17$
Post-synaptic membrane length (μm)	$31\cdot25\pm2\cdot43$	$23\cdot98\pm2\cdot55$	$28\cdot92\pm2\cdot60$
Post-synaptic membrane length/nerve terminal length	$5\cdot49\pm0\cdot29$	$5\cdot19\pm0\cdot36$	$9\cdot50\pm1\cdot33*$
Post-synaptic area/nerve terminal area	$1\cdot67\pm0\cdot25$	$1\cdot78\pm0\cdot49$	$4\cdot95\pm1\cdot08*$

All data are expressed as mean$\pm$s.e. of mean and are derived from longitudinally sectioned end-plates only.

* Young and old significantly different ($P < 0\cdot001$).

Investigation of possible denervation

Given the morphologic finding that many post-synaptic regions in old e.d.l. muscles showed absent nerve terminals, we sought additional evidence for chronic denervation and reinnervation:

(*a*) After reinnervation of denervated muscle, polyaxonal innervation of muscle often persists for about 1 month (Gorio, Carmignoto, Facci & Finesso, 1980; McArdle, 1975). However, in a total of ninety-five old sol. muscle fibres from nine mice, only nine showed physiological evidence of polyaxonal innervation (cf. Redfern, 1970). In old e.d.l. muscles, the incidence was 25 of 153 fibres from sixteen mice.

(*b*) If denervation and reinnervation were widespread in old muscles, smaller axons with longer nerve refractory periods should be prevalent, but this was not the case.

(*c*) Impalement of randomly chosen uncurarized muscle fibres in young and old (34 months) sol. and e.d.l. revealed no substantial difference in the percentage of fibres that failed to produce an action potential in response to nerve stimulation.

(*d*) Cross-sections of sol. and e.d.l. muscles stained for succinic dehydrogenase and acid-stable ATPase (Pearse, 1972) were examined for evidence of the 'type-grouping' and group atrophy characteristic of a chronic denervation-reinnervation process

(Karpati & Engel, 1968). In five old mice, no group atrophy was present, and only one animal showed any suggestion of 'type-grouping' (in both e.d.l. and sol.).

(*e*) In the few cases studied, counts of total numbers of muscle fibres in complete cross-sections showed no change with age in e.d.l. (910 fibres young *vs*. 1006 fibres, old) or sol. (843 fibres young *vs*. 801 fibres old), in two young and four old muscles.

(*f*) Indirect isometric twitch tension of old sol. (2.5 ± 0.2 g, $n = 6$) was not significantly different from that of young sol. (3.3 ± 0.3 g, $n = 7$).

(*g*) In all cases where junctional ^{125}I-α-bungarotoxin binding was measured (Table 1), extrajunctional binding in non-end-plate muscle segments was no greater than background.

DISCUSSION

M.e.p.p. frequency

One objective of this study was to extend the study of age-related changes at the neuromuscular junction to the mouse, previous studies having been mostly confined to the rat. However, the development state of the control 'young' animals must be considered first. Use of m.e.p.p. frequency data from 3 month rat muscle (Vyskocil & Gutmann, 1972) is tenous because m.e.p.p. frequency is increasing at that age, reaching a plateau by 6 months (Kelly, 1978). The present study of mouse diaph. ('young' controls 8–11 months of age) and Smith's (1979) result in rat diaph. ('young' controls 12–13 months of age) both demonstrate little or no change in m.e.p.p. frequency with age in that muscle. In the sol. muscle of both species, m.e.p.p. frequency consistently decreased with age (compare present results with Gutmann *et al.* 1971; Vyskocil & Gutmann, 1972). We found no age change in m.e.p.p. frequency in flexor digitorum brevis, whereas, in the e.d.l., we observed a decrease at 28–33 months and a return to control levels at 34 months. Thus, reduction in m.e.p.p. frequency with age is not a universal finding in distal fast twitch limb muscles.

In light of this data several hypotheses about age-related nerve terminal changes can be evaluated. First, rostro-caudal progression of change (Fujisawa, 1976) is ruled out because the cervically innervated e.d.c. is affected while the diaphragm is not, and the flexor digitorum brevis, innervated from the lower lumber cord, is also unaffected. A second hypothesis, that muscle activity determines the results, must at least be modified. If total limb activity were reduced, one would expect changes in both the g.m. and in other limb muscles, but this was not the case. However, the more pronounced effects in the tonic sol. than in e.d.l. or e.d.c., which are probably phasic, suggest that inactivity may differentially affect those muscles which normally fire continuously. The diaphragm, then, would be spared because of its obligatory respiratory function. A third hypothesis is that diminished axonal transport (Komiya, 1980) leads to deficits in nerve terminals supplied by the longest axons. However, there was no correlation between length of axon and decrease in m.e.p.p. frequency. This suggests that simple distance from the cell body is not a major factor.

The mechanism of decrease in m.e.p.p. frequency in aged nerve terminals is not established. No corresponding reduction in cholinesterase-positive end-plate area was found. Reduced number of available synaptic vesicles (present results; Fahim & Robbins, 1982), fewer active zones per nerve terminal length, or an altered release mechanism are all possibilities.

M.e.p.p. amplitude

Only the diaphragm in old mice revealed altered m.e.p.p. amplitudes with age, and these became large than in young mice. This was not associated with any significant change in muscle fibre diameter, input resistance, or membrane capacitance. M.e.p.p. rise and decay time and over-all end-plate ACh receptor density were also unchanged and there was only a small change in resting potential. Therefore, other explanations for the increased m.e.p.p. amplitude, e.g. involving the number of ACh molecules released or the post-synaptic response per quantum, require further investigation. Some alterations may also occur in other old muscles where m.e.p.p. amplitude was not changed despite significantly increased input resistance or reduced acetylcholine receptors. In contrast to amphibian muscle, input resistance in rodent muscle would affect m.e.p.p. amplitude (Gage & McBurney, 1973) because of the relatively low specific membrane resistance (e.g. Albuquerque & Thesleff, 1968).

Evoked transmitter release

In evaluating synaptic transmission at young and old neuromuscular junctions, we chose three different methods of blocking the twitch. In no instance was synaptic transmission reduced with advanced age: it was either the same or greater. This is the most important physiological conclusion of this work.

Could the use of (+)-tubocurarine (curare) have influenced the outcome? First, the larger e.p.p. amplitude in curarized aged e.d.l. and sol. muscles could indicate diminished pre- or post-synaptic curare sensitivity, but the relative e.p.p. amplitudes (young *vs.* old) were proportional to the Poisson-calculated quantum content, which would not be expected according to this hypothesis. Secondly, it was found that e.p.p. amplitudes in cut fibre preparations were equally reduced by curare in young and old muscle fibres. Thirdly, in another study (Gertler & Robbins, 1978), *relative* differences in synaptic transmitter output were preserved in the presence and absence of curare. Fourthly, the results from preparations in Krebs solutions with low calcium:magnesium ratio were in complete accord with the curare experiments. In sum, it is likely that some of the absolute values presented here may be altered by the effects of curare, but that the comparisons between young and old junctions are still valid.

At both 2 Hz and 20 Hz, e.p.p. amplitudes and safety factors in old junctions were either greater than (e.d.l., sol.) or equal to (diaph.) those in young junctions. In e.d.l. and sol., m.e.p.p. amplitudes, resting potential and spike threshold were similar in young and old mice. Therefore, in these muscles an increase in quantum content apparently increases the safety factor.

Age differences in the 'rundown' of amplitudes of the first several e.p.p.s in a train appear to be absent (e.d.l., diaph.) or of little significance (sol.). Our results in diaph. differ from those reported in old rats (Smith, 1979), in which the second e.p.p. of a pair was considerably depressed over a wide range of intervals.

In the diaph. the similar safety factor in young and old muscle mainly derived from the larger m.e.p.p. amplitude in old animals, and to a lesser extent a small threshold change. Surprisingly, however, both m.e.p.p. amplitude and the Poisson-computed quantal content were equal in young and old diaph. even though the m.e.p.p.

amplitude was unequal. A number of changes in the junctional physiology of old diaph. could account for this apparent discrepancy. They include altered release statistics, diminished 'unitary' size during tetani compared to spontaneous m.e.p.p. amplitude, or altered post-synaptic sensitivity to curare.

Cut-fibre preparations showed no age-dependent differences in e.p.p. amplitude or direct quantal content. However some unusual findings in these preparations limit their heuristic value. Because of lower resting potential, fibres with larger m.e.p.p. amplitudes or those non-twitching fibres with lower safety factors may have been selected. The absence of response in some 10 % of fibres impaled in end-plate regions (*vs.* a much lower percentage in curarized preparations) indicates some presynaptic blockade, as previously reported in cut-fibre preparations (Hubbard & Wilson, 1973). Another unusual feature of this preparation was the low quantal content, yielding computed safety factors less than or close to 1·0 whether voltage recording (present study) or voltage clamp (Glavinovic, 1979) was employed.

The disparity in age-related results between curarized or Ca-deprived preparations on the one hand and cut fibre preparations on the other may result from problems of selection or presynaptic factors.

In 34 month animals, nerve terminal area was reduced more than two-fold, with corresponding loss of synaptic vesicle number as well as a reduction in synaptic vesicle density. Despite this three-fold reduction in total number of vesicles per junction, the safety factor was higher than in young animals. A similar disparity was present in e.d.l. and sol. muscle from 29–30 month mice, where increased e.p.p. amplitudes (and less indirect twitch depression in solutions with reduced calcium) were observed despite a threefold reduction in vesicle density (present ultrastructural results in e.d.l.; also Fahim & Robbins, 1982). Although there is not always good correlation between number of synaptic vesicles and transmitter release (Ceccarelli & Hurlbut, 1980), it is generally true that vesicle depletion is associated with transmitter reduction or failure (Ceccarelli, Hurlbut & Mauro, 1972; Heuser & Miledi, 1971; Jones & Kwanbunbumpen, 1970). One explanation would be that only a certain fraction are released during normal physiological activity (McLachlan, 1978), and these are preserved in aging junctions. Alternatively, vesicles may recycle more rapidly in the aged neuromuscular junction, thereby achieving the high safety factor of the plateau e.p.p. Finally, the limitation on quantal content may be the number of release sites rather than number of vesicles. In this event, loss of $\frac{2}{3}$ of the normal vesicle content would have little effect on short term tetanic release (see below), or may be countered by increased nerve terminal branching, yielding more release sites (Fahim, Holley & Robbins, 1982).

The uniformity of the age-dependent physiological findings in different fibres of the same e.d.l. muscle contrasts with the heterogeneity of certain ultrastructural findings. For instance, every synaptic region in three of fifteen junctions from 30 months old mouse e.d.l. showed massive engorgement with neurofilaments, and seven of 20–34 month old junctions had autophagic vacuoles. Yet, extreme physiological synaptic deficit was not characteristic of *any* e.d.l. fibres examined in these age groups. Therefore, these morphological changes do not imply altered nerve terminal performance. Eight out of twenty synapses at 34 months showed whole synaptic regions with absent nerve terminals yet failure of the indirect action potential in 34 month

muscle was no greater than in young animals where terminals were invariably present. Thus, partial withdrawal of nerve terminals from some regions of a synapse does not proceed to denervation. Indeed, e.p.p. amplitude data in 34 month e.d.l. was not different from that in 30 month animals even though there were more denuded postsynaptic sites and smaller nerve terminals. At such junctions, increased output might result if the density or associated probabilities of release sites were increased in other terminals of the same junction, especially since heterogeneity in numbers of functional release sites of the terminals of a single motor axon has been reported (Bennett & Lavidis, 1979; Meiss & Govind, 1980). Finally, m.e.p.p. frequency and quantal content are often correlated (Grinell & Herrera, 1980), but this was not the case in old e.d.l. and sol., where decreased m.e.p.p. frequency was associated with increased evoked release. Possibly, the reduced total numbers of synaptic vesicles in junctions of old e.d.l. and sol. bring about this dissociation between spontaneous and evoked release.

In sum, the ageing synapse appears to be highly compensated and *different* from rather than simply deteriorated compared to that of a young animal. The differing sets of compensations in different muscle groups may have to do with the initial characteristics of junctions adapted to different daily firing patterns (Robbins, 1980). Thus, increased numbers of release sites may explain the greater quantal release in old sol. neuromuscular junctions, whereas altered receptor mechanism or acetylcholine per quantum may explain the normal m.e.p.p. amplitude in e.d.l. despite reduced junctional receptors.

Evidence of denervation and reinnervation

It has often been suggested that aged neuromuscular junctions are continuously undergoing degeneration and regeneration (e.g. Gutmann & Hanzlikova, 1965). Both the ectopic end-plate location in aged rat soleus (Gutmann & Hanzlikova, 1965) and the presence of 'type-grouping' in certain aged human muscles (Jennekens, Tomlinson & Walton, 1971) are consistent with this interpretation. However, in the aged mouse, only single end-plates were found on all muscle fibres examined and type-grouping was not observed. In addition, there were few hallmarks of complete denervation: muscle fibre diameters were normal in size, extrajunctional acetylcholine receptors were absent, and rise and decay times of m.e.p.p.s were normal, so that cholinesterase activity was approximately normal as well. Therefore, it is likely that those changes found that are common to denervation, namely decrease in resting membrane potential and increase in input resistance, are age-related phenomena rather than sequelae of denervation. Signs of overt denervation in some other species (Gutmann & Hanzlikova, 1965; Gutmann et al. 1971; Jennekins et al. 1971; Caccia et al. 1979) may be later stages of the same processes found in aged mice, or a more extreme case exacerbated by inactivity or undetected pathology.

The reduction in *junctional* ACh receptors characteristic of prolonged denervation (Frank, Gautrik & Sommerschild, 1975) was only found in one of the five muscles examined (e.d.l.) but the normal m.e.p.p. amplitude found in the same muscle suggests that the reduced receptors were not in functional locations or were compensated by other factors (e.g. input resistance). Also, the maintenance with age of normal numbers of junctional receptors in four of five muscle types, agrees with

results in rat soleus (Pestronk *et al.* 1980), and suggests that a recently reported reduction in rat sternomastoid receptors in old animals (Courtney & Steinbach, 1981) is not a general finding.

REFERENCES

ALBUQUERQUE, E. X. & THESLEFF, S. (1968). A comparative study of membrane properties of innervated and chronically denervated fast and slow skeletal muscles of the rat. *Acta physiol. scand.* **73**, 471–480.

BENNETT, M. R. & LAVIDIS, N. A. (1979). The effect of calcium ions on the secretion of quanta evoked by an impulse at nerve terminal release sites. *J. gen. Physiol.* **74**, 429–456.

CACCIA, M. R., HARRIS, J. B. & JOHNSON, M. S. (1979). Morphology and physiology of skeletal muscle in aging rodents. *Muscle Nerve* **2**, 202–212.

CECCARELLI, B. & HURLBUT, W. P. (1980). Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* **60**, 396–441.

CECCARELLI, B., HURLBUT, W. P. & MAURO, A. (1972). Depletion of vesicles from frog neuro-muscular junctions by prolonged tetanic stimulation. *J. cell. Biol.* **54**, 30–38.

COURTNEY, J. & STEINBACH, J. H. (1981). Age changes in neuromuscular junction morphology and acetylcholine receptor distribution on rat skeletal muscle fibres. *J. Physiol.* **320**, 435–447.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.* **24**, 560–573.

ENGEL, A. G., TSUJIHATA, M. & JERUSALEM, F. (1975). Quantitative assessment of motor endplate ultrastructure in normal and diseased human muscle. In *Peripheral Neuropathy*, ed. DYCK, P. J., THOMAS, P. K. & LAMBERT, E. H., vol. II, chap. 69, pp. 1404–1415. Philadelphia: W. B. Saunders Company.

FAHIM, M. A., HOLLEY, J. A. & ROBBINS, N. (1982). Scanning and light microscopic study of age changes at a neuromuscular junction in the mouse. *J. Neurocytol.* (in the Press.)

FAHIM, M. A. & ROBBINS, N. (1982). Ultrastructural studies of young and old mouse neuromuscular junctions. *J. Neurocytol.* **11**, 641–656.

FAMBROUGH, D. (1979). Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* **59**, 165–227.

FISCHER, M. B. & BIRREN, J. E. (1947). Age and strength. *J. appl. Physiol.* **31**, 490–497.

FRANK, E., GAUTRIK, K. & SOMMERSCHILD, H. (1975). Cholinergic receptors at denervated mammalian motor endplates. *Acta physiol. scand.* **95**, 66–76.

FUJISAWA, K. (1976). Some observations on the skeletal musculature of aged rats. III. Abnormalities of terminal axons found in motor endplates. *Exp. Gerontol.* **11**, 43–47.

GAGE, P. W. & McBURNEY, R. N. (1973). An analysis of the relationship between the current and potential generated by a quantum of acetylcholine in muscle fibers without transverse tubules. *J. Membrane Biol.* **12**, 247–272.

GERTLER, R. & ROBBINS, N. (1978). Differences in neuromuscular transmission in red and white muscles. *Brain Res.* **142**, 160–164.

GLAVINOVIC, M. I. (1979). Voltage clamping of unparalysed cut rat diaphragm for study of transmitter release. *J. Physiol.* **290**, 467–480.

GORIO, A., CARMIGNOTO, G., FACCI, L. & FINESSO, M. (1980). Motor nerve sprouting induced by ganglioside treatment. Possible implications for gangliosides on neuronal growth. *Brain Res.* **197**, 236–241.

GRINNELL, A. D. & HERRERA, A. A. (1980). Physiological regulation of synaptic effectiveness at frog neuromuscular junctions. *J. Physiol.* **307**, 301–318.

GRINNELL, A. D. & HERRERA, A. A. (1981). Specificity and plasticity of neuromuscular connections: long-term regulation of motoneuron functions. *Prog. Neurobiol.* **17**, 203–282.

GUTMANN, E. & HANZLIKOVA, V. (1965). Age changes of motor endplates in muscle fibres of the rat. *Gerontologia* **11**, 12–24.

GUTMANN, E., HANZLIKOVA, V. & VYSKOCIL, F. (1971). Age changes in cross striated muscle of the rat. *J. Physiol.* **219**, 331–343.

HARRIS, J. B. & RIBCHESTER, R. R. (1979). The relationship between end-plate size and transmitter release in normal and dystrophic muscle of the mouse. *J. Physiol.* **296**, 245–265.

HEUSER, J. & MILEDI, R. (1971). Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc.* B **179**, 247–260.

HUBBARD, J. I. & WILSON, D. F. (1973). Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of D-tubocurarine. *J. Physiol.* **228**, 307–325.

JENNEKENS, F. G. I., TOMLINSON, B. E. & WALTON, J. N. (1971). Histochemical aspects of five limb muscles in old age. *J. Neurol. Sci.* **144**, 259–276.

JONES, S. F. & KWANBUNBUMPEN, S. (1970). The effects of nerve stimulation and hemicholinium on synaptic vesicles at the mammalian neuromuscular junction. *J. Physiol.* **207**, 31–50.

KARPATI, G. & ENGLE, W. K. (1968). 'Type grouping' in skeletal muscles after experimental reinnervation. *Neurology Minneap.* **18**, 447–455.

KATZ, B. & THESLEFF, S. (1957). On the factors which determine the amplitude of the 'miniature end-plate potential'. *J. Physiol.* **137**, 267–278.

KELLY, S. S. (1976). The effects of age and nutritional state on some parameters of neuromuscular transmission in the rat. Ph.D. Thesis, Liverpool University.

KELLY, S. S. (1978). The effects of age on neuromuscular transmission. *J. Physiol.* **274**, 51–62.

KOHN, R. R. (1978). *Principles of Mammalian Aging.* Inglewood Cliffs: Prentiss-Hall Inc.

KOMIYA, Y. (1980). Slowing with age of the rate of slow axonal flow in biforcating axons of rat dorsal root ganglion cells. *Brain Res.* **183**, 477–480.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–122.

McARDLE, J. J. (1975). Complex end-plate potentials at the regenerating neuromuscular junction of the rat. *Expl Neurol.* **49**, 629–638.

McLACHLAN, E. M. (1978). The statistics of transmitter release at chemical synapses. *Int. Rev. Physiol.* **17**, 49–117.

McLACHLAN, E. M. & MARTIN, A. R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol.* **311**, 307–324.

MEISS, D. E. & GOVIND, C. K. (1980). Heterogeneity of excitatory synapses at the ends of single muscle fibres in lobster, *Homarus americanus. J. Neurobiol.* **11**, 381–395.

PEARSE, A. G. E. (1972). *Histochemistry: Theoretical and Applied*, vol. 2, Baltimore: Williams & Williams.

PESTRONK, A., DRACHMAN, D. B. & GRIFFIN, J. W. (1980). Effects of aging on nerve sprouting and regeneration. *Expl Neurol.* **70**, 65–82.

REDFERN, P. A. (1970). Neuromuscular transmission in newborn rats. *J. Physiol.* **209**, 701–709.

ROBBINS, N. (1980). Plasticity of mature neuromuscular junction. *Trends in Neurosci.* **5**, 120–122.

ROBBINS, N., OLEK, A., KELLY, S. S., TAKACH, P. & CHRISTOPHER, M. (1980). Quantitative study of motor endplates in muscle fibres dissociated by a simple procedure. *Proc. R. Soc.* B **209**, 555–562.

RUSSEL, E. S. (1972). Genetic considerations in the selection of rodent species and strains for research in aging. In *Development of the Rodent as a Model System of Aging* ed. GIBSON, D. C., pp. 33–53. U.S. Dept. Health, Education, and Welfare Publication No. (NIH) 72–121.

SMITH, D. O. (1979). Reduced capabilities of synaptic transmission in aged rats. *Expl Neurol.* **66**, 650–666.

SNEDECOR, G. W. & COCHRAN, W. G. (1980). *Statistical Methods*, pp. 291–292, Ames, IA: Iowa State University Press.

STANDAERT, F. G. & BOOHER, K. (1981). Neuromuscular blocking drugs. In *CRC Handbook of Physiology in Aging*, ed. MASORO, E. J. Boca Raton: CRC Press, Inc.

VYSKOCIL, F. & GUTMANN, E. (1972). Spontaneous transmitter release from nerve endings and contractile properties in the soleus and diaphragm muscles of senile rats. *Experientia* **28**, 280–281.

B. Q. BANKER, S. S. KELLY AND N. ROBBINS
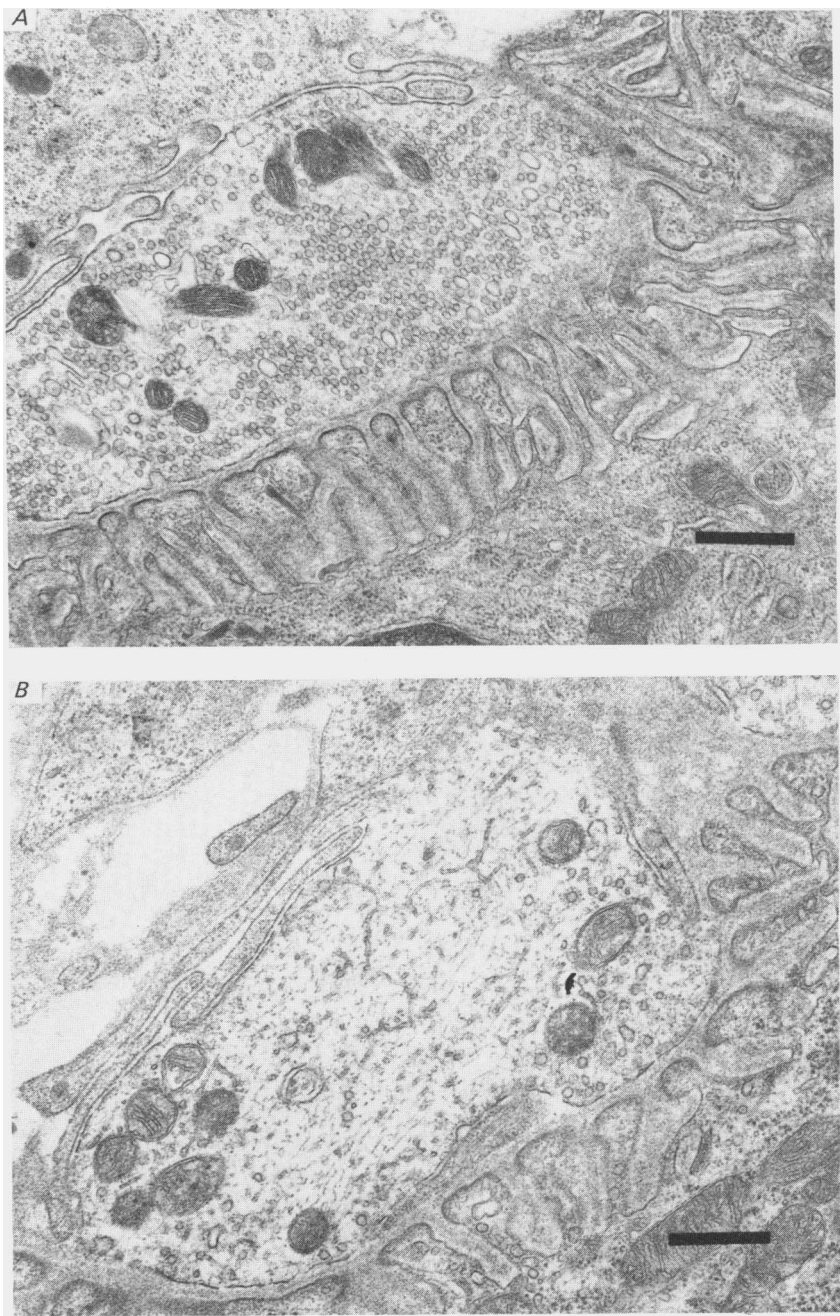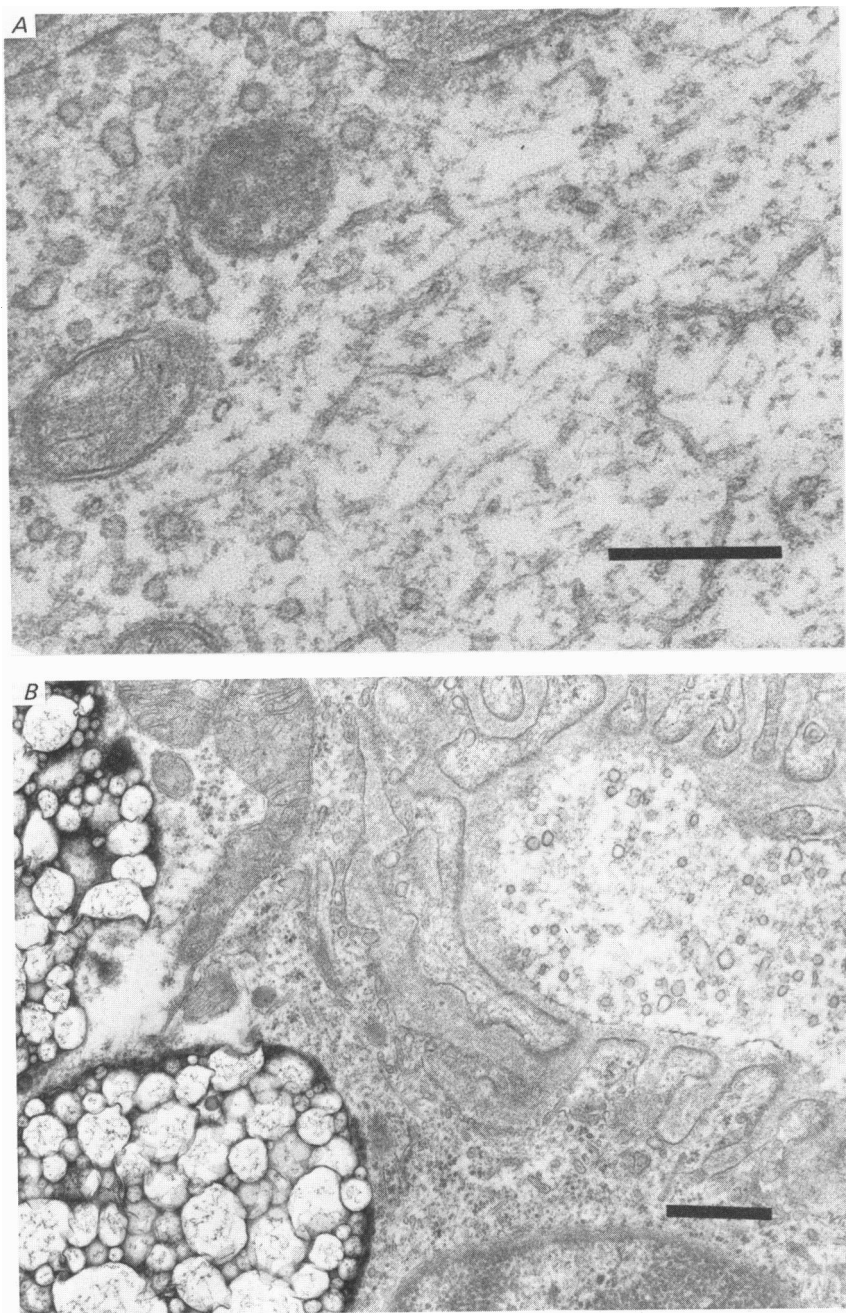
EXPLANATION OF PLATES

PLATE 1

Motor end-plate region of extensor digitorum longus muscle in a young adult (*A*) and in 30 month old (*B*) CBF-mouse. Calibration bar: 500 nm. Note depletion of synaptic vesicles and conspicuous neurotubules and neurofilaments in *B* compared to *A*.

PLATE 2

A, a higher magnification of the nerve terminal in Fig. 7, demonstrating the prominence of fibrillar and tubular elements in nerve terminals of old CBF-1 extensor digitorum longus muscles. Calibration bar: 300 nm. *B*, motor end-plate region of e.d.l. muscle in the old CBF-1 mouse. Calibration bar: 500 nm. As in Pl. 1*B*, synaptic vesicles are depleted and filamentous material is abundant. In addition, there is accumulation of lipofuchsin in close proximity to the synaptic area.